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Metastatic breast tumor Met-1 cells express $CD44v_{3,8\text{-}10}$, a major adhesion receptor which binds extracellular matrix components at its extracellular domain and interacts with the cytoskeletal protein, ankyrin, at its cytoplasmic domain. In this study we have determined that $CD44v_{3,8\text{-}10}$ and RhoA GTPases are physically associated in vivo, and that $CD44v_{3,8\text{-}10}$-bound RhoA displays GTPase activity which can be inhibited by botulinum toxin C3-mediated ADP-ribosylation. In addition, we have identified a 160kDa Rho-Kinase (ROK) as one of the downstream targets for $CD44v_{3,8\text{-}10}$-bound RhoA GTPase. Specifically, RhoA (complexed with $CD44v_{3,8\text{-}10}$) stimulates ROK-mediated phosphorylation of certain cellular proteins including the cytoplasmic domain of $CD44v_{3,8\text{-}10}$. Most importantly, phosphorylation of $CD44v_{3,8\text{-}10}$ by ROK enhances its interaction with the cytoskeletal protein, ankyrin.

We have also constructed two ROK cDNA constructs which encode for proteins consisting of 537 amino acids [designated as the constitutively active form of ROK containing the catalytic domain (CAT, also the kinase domain)], and 173 amino acids [designated as the dominant-negative form of ROK containing the Rho-binding domain (RB)]. Microinjection of the ROK's CAT domain into Met-1 cells promotes CD44-ankyrin associated membrane ruffling and projections. This membrane motility can be blocked by CD44 antibodies and cytochalasin D (a microfilament inhibitor). Furthermore, overexpression of a dominant-negative form of ROK by transfection of Met-1 cells with ROK's Rho-binding (RB) domain cDNA effectively inhibits CD44-ankyrin-mediated metastatic behavior (e.g. membrane motility and tumor cell migration). These findings support the hypothesis that ROK plays a pivotal role in $CD44v_{3,8\text{-}10}$-ankyrin interaction and RhoA-mediated oncogenic signaling required for membrane-cytoskeleton function and metastatic tumor cell migration.

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INTRODUCTION

A number of studies have been aimed at identifying molecules expressed by breast tumor cells which correlate with metastatic behavior. One such candidate is CD44 which belongs to a family of transmembrane glycoproteins. Specifically, CD44s (the standard form, molecular mass of 85-95 kDa) is widely expressed on hematopoietic cells, fibroblasts, and certain transformed epithelial cells (Bourguignon et al., 1986; Bourguignon et al., 1997; Green et al., 1988; Iida and Bourguignon, 1995; Iida and Bourguignon, 1997; Kalomiris and Bourguignon, 1988; Lesley et al., 1985; Letarte 1985; Stamenkovic et al., 1991; Zhu and Bourguignon, 1996). A larger protein, CD44E (the epithelial form, molecular mass of 125-150 kDa), results from the alternative splicing of three additional exons [exons 12-14 (v8-10)] into the membrane proximal region of the CD44 molecule and is preferentially expressed on epithelial cells (Screaton, 1992; Stamenkovic et al., 1991). Higher molecular weight "variant" isoforms of CD44 (CD44v) are derived from the alternative splicing of up to ten additional exons in various combinations of the extracellular domain of the molecule (Screaton et al., 1992). Cell surface expression of certain CD44v isoforms (e.g. CD44v3-containing isoforms) appears to change profoundly during tumor metastasis-particularly during the progression of various carcinomas including breast carcinomas (Arch et al., 1992; Bennet et al., 1995; Droll et al., 1995; Gunthert et al., 1991; Herrilich et al., 1993; Hofmann et al., 1991; Iida and Bourguignon, 1995; Welsh et al., 1995). Furthermore, CD44v isoforms have been detected on highly metastatic cell lines and transfection of these molecules confers metastatic properties on otherwise non-metastatic cells (Hofmann et al., 1991; Iida and Bourguignon, 1997).

All CD44 isoforms (e.g. CD44s, CD44E and CD44v) contain several hyaluronic acid (HA)-binding sites in their extracellular domain (Bourguignon et al., 1992; Bourguignon et al., 1993; Bourguignon, 1995; Lesley et al., 1985; Lokeshwar et al., 1991). The binding of CD44 isoforms to HA causes cell adhesion to the extracellular matrix (ECM) components (Bourguignon et al., 1992; Bourguignon et al., 1993; Bourguignon, 1995; Lesley et al., 1993; Lokeshwar and Bourguignon, 1991) and has also been implicated in the stimulation of cell proliferation, cell migration and angiogenesis (Lokeshwar et al., 1996; Rooney et al., 1995; Turley et al.,1991; West and Kumar, 1989). The intracellular domain of CD44 binds to certain cytoskeletal proteins such as ankyrin (Bourguignon et al., 1991; Bourguignon et al., 1992; Bourguignon, et al., 1993, Bourguignon et al., 1994; Bourguignon, 1995; Bourguignon et al., 1998; Kalomiris and Bourguignon, 1988; Kalomiris and Bourguignon, 1989; Lokeshwar and Bourguignon, 1991; Lokeshwar and Bourguignon, 1992; Lokeshwar et al., 1994; Lokeshwar et al., 1996; Zhu and Bourguignon, 1996; Zhu and Bourguignon, 1998) and ERM proteins (exrin, radixin and moesin) (Tsukita et al., 1994). Post-translational modification of CD44's cytoplasmic domain by either acylation (Bourguignon et al., 1991), protein kinase C-mediated phosphorylation (Lokeshwar and Bourguignon, 1992; Kalomiris and Bourguignon, 1989), or GTP binding (Iida and Bourguignon, 1995; Kalomiris and Bourguignon, 1988) enhances the binding between CD44 and cytoskeletal proteins. The transmembrane interaction between CD44 isoforms and ankyrin/ERM provides a direct link between the ECM and the cytoskeleton. A recent study has shown that CD44v isoforms, such as $CD44v_{3,8\text{-}10}$, are involved in cytoskeleton-mediated breast tumor cell migration and invasion (Bourguignon et al., 1998). However, very little is presently known concerning the CD44v3-associated oncogenic signaling cascade which results in the metastatic phenotype of breast tumor cells.

Members of the Rho subclass of the ras superfamily [small molecular weight GTPases, (e.g. RhoA, Rac1 and Cdc42)] are known to be associated with changes in the membrane-linked cytoskeleton (Hall, 1998; Narumiya, 1996). For example, activation of RhoA, Rac1 and Cdc42 have been shown to produce specific structural changes in the plasma membrane-cytoskeleton associated with membrane ruffling, lamellipodia, filopodia, and stress fiber formation (Hall, 1998; Narumiya, 1996). The coordinated activation of these GTPases is considered to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis (Dickson and Lippmann, 1995; Jiang et al., 1994; Lauffenburger and Horwitz, 1996). Tsukita and his co-workers have reported that Rho-like proteins participate in the interaction between the CD44 and the ERM cytoskeletal proteins (Hirao et al., 1996). The question whether Rho-like proteins play a direct role in regulating $CD44v_3$-specific metastatic behaviors in breast tumor cells is addressed in this study.

Several enzymes have been identified as possible downstream targets for Rho GTPases in regulating cytoskeleton-mediated cell motility. One such enzyme is Rho-Kinase (ROK-also called Rho-binding kinase) which is a serine-threonine kinase known to interact with Rho in a GTP-dependent manner (Hall, 1998; Matsui et al., 1996). Structurally, ROK is composed of catalytic (CAT), coiled-coil, Rho-binding (RB) and pleckstrin-homology (PH) domains (Hall, 1998; Matsui et al., 1996). Expression of the catalytic domain alone by deleting the regulatory domain [e.g. Rho-binding (RB) domain] causes ROK constitutively active (Amano et al., 1997). Therefore, the catalytic fragments may act as dominant active forms and the Rho-binding fragments can function as dominant negative form of the ROK molecule (Amano et al., 1997). Currently, two substrates, myosin light chain and myosin light chain phosphatase, have been shown to be phosphorylated by ROK (Amano et al., 1996, Kimura et al., 1996). Importantly, ROK-mediated phosphorylation of these two proteins activates myosin adenosine triphosphatase (ATPase) leading to actomyosin-mediated membrane motility and cell movement (Amano et al., 1996; Amano et al., 1997; Kimura et al., 1996). This information has prompted us to examine whether ROK plays a role in regulating $CD44v_{3,8-10}$-mediated cytoskeleton function in metastatic tumor cells.

In this paper, using a variety of biochemical and molecular biological techniques, we have found that the cell adhesion receptor, $CD44v_{3,8-10}$ is preferentially expressed in Met-1 breast tumor cells (derived from a high metastatic potential tumor in transgenic mice expressing polyomavirus middle T oncogene). In addition, $CD44v_{3,8-10}$ is found to be physically linked to RhoA GTPase in Met-1 cells. We have also demonstrated that RhoA (complexed with $CD44v_{3,8-10}$) stimulates Rho-Kinase (ROK) [a known downstream target for RhoA] to phosphorylate several cellular proteins including $CD44v_{3,8-10}$. Most importantly, the phosphorylation of $CD44v_{3,8-10}$ by ROK promotes the binding of $CD44v_{3,8-10}$ to ankyrin. Overexpression of the Rho-binding (RB) domain (a dominant-negative form) of ROK by transfecting Met-1 cells with RB cDNA induces reversal of tumor cell-specific phenotypes. Therefore, we believe that $CD44v_{3,8-10}$ and RhoA-mediated signaling is involved in the up-regulation of ROK needed for membrane-cytoskeleton interaction and tumor cell migration during the progression of metastatic breast tumor cells.

BODY

MATERIALS AND METHODS

Cell Culture: Mammary tumor cells containing the polyoma virus middle T (PyV-MT) transgene under the transcriptional control of the MMTV LTR promoter were used to initiate a transplantable line in nude mice. The PyV-MT transgenic mammary tumor cells were obtained from mammary tumors which arose in the transgenic colony at the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada (Dr. Willliam J. Muller) (Guy et al., 1992). Mammary tumors were collagenase/dispase (Worthington, Freehold, N. J.) treated, and 5×10^5 cells per 100µl were transplanted subcutaneously as a bolus via syringe and a 25 gauge needle into the thoracic region of nude mice. The resulting high potential metastatic PyV-MT transgenic mammary tumor line, Met-1, was maintained by serial tansplantation of 1 mm^3 tumor segments into either subcutaneous tissue (ectopic) or intact mammary fat pads (orthotopic).

The Met-1 tumor line was dissociated after transplant generation one and plated onto T-75 flasks to develop a tissue culture line (Cheung et al., 1997). The Met-1 cell line was cultured in high glucose DMEM supplemented by 10% fetal bovine serum, 2mM glutamine, and antibiotics (Sigma, St Louis, MO).

Antibodies and Reagents: Monoclonal rat anti-human CD44 antibody (Clone:020; Isotype: IgG_{2b}; obtained from CMB-TECH, Inc., Miami, FL.) used in this study recognizes a common determinant of the CD44 class of glycoproteins including CD44s and other variant isoforms (Iida and Bourguignon) and is capable of precipitating all CD44 variants. For the preparation of polyclonal rabbit anti-CD44v3 or rabbit anti-Rho-Kinase (ROK) antibody, specific synthetic peptides [≈15-17 amino acids unique for either CD44v3 or N-terminus ROK] were prepared, respectively by the Peptide Laboratories of Department of Biochemistry and Molecular Biology at the University of Miami Medical School using an Advanced Chemtech automatic synthesizer (model ACT350). Conjugated CD44v3 or ROK peptides (to polylysine) were injected into rabbits to raise the antibodies. All antibodies (e.g. anti-CD44v3 or anti-ROK sera) were collected from each bleed and stored at 4°C containing 0.1% azide. All antibodies (e.g. rabbit anti-CD44v3 IgG or rabbit anti-ROK IgGs) were prepared using conventional DEAE-cellulose chromatography and were tested to be monospecific (by immunoblot assays). Mouse monoclonal

ankyrin (Ank1) antibody was prepared as described previously (Bourguignon et al., 1993). Rabbit anti-Ank3 antibody was kindly provided by Dr. L. L. Peters (Jackson Laboratory, Bar Harbor, ME) (Peters, et al., 1995). Mouse monoclonal anti-green fluorescent protein (GFP) and anti-glutathione S-transferase (GST) were purchased from PharMingen and Pharmacia, respectively. Rhodamine-labeled phalloidin was purchase from Molecular Probes, Inc. Clostridium botulinum C3 toxin was obtained from List Biological Laboratories, Inc. Escherichia coli-derived GST-tagged RhoA was a gift from Dr. Martin Schwartz (Scripps Research Institute, La Jolla, CA).

Cloning, Expression and Purification of CD44 Cytoplasmic Domain (CD44cyt) from E. coli: The cytoplasmic domain of human CD44 (CD44cyt) was cloned into pFLAG-AST using the PCR-based cloning strategy. Using human CD44 cDNA as template, one PCR primer pair (left, FLAG-EcoRI; right, FLAG-XbaI) was designed to amplify complete CD44 cytoplasmic domain. The amplified DNA fragments were one-step cloned into a pCR2.1 vector and sequenced. Then, the DNA fragments were cut out by double digestion with EcoRI and XbaI and subcloned into EcoRI/XbaI double-digested pFLAG-AST (Eastman Kodak Co.-IBI, Rochester, NY) to generate FLAG-pCD44cyt construct. The nucleotide sequence of FLAG/CD44cyt junction was confirmed by sequencing. The recombinant plasmids were transformed to BL21-DE3 to produce FLAG-CD44cyt fusion protein. The FLAG-CD44cyt fusion protein was further purified by anti-FLAG M2 affinity gel column (Eastman Kodak Co.-IBI, Rochester, NY). The nucleotide sequence
of primers used in this cloning protocol are:

FLAG-EcoRI: 5'-GAGAATTCGAACAGTCGAAGAAGGTGTCTCTTAAGC-3';
FLAG-XbaI: 5'-AGCTCTAGATTACACCCCAATCTTCAT-3'.

Preparations of Constitutively Active Form and Dominant-Negative Form of Rho-Kinase (ROK):

(a) Method for preparing GST-tagged constitutively active form [containing catalytic domain (CAT)] of Rho-Kinase (ROK): The cDNA fragment encoding the constitutively active form of ROK [1-1611bp, containing the catalytic domain (CAT)] was generated by reverse transcription-polymerase chain reaction (RT-PCR) using CAT-specific primers, 5'-GACGACGACAAGATGTCGACTGGGGACAGTTTTGAGAC-3' and 5'-CAGGACAGA GCATCAATTAGCAAGCTGTGAATTCTGACT-3'. These cDNA fragments were then cloned into pESP-2 vector using ESP LIC Cloning Kit (Stratagene) to produce GST-CAT construct. The inserted CAT sequence was verified by nucleotide sequencing analyses. Subsequently, this GST-CAT cDNA was introduced to an eukaryotic expression system such as yeast Schizosaccharomyces pombe (Kohli, 1987) to express GST-CAT fusion protein (M.W. 80 kDa). The fusion protein was purified by a glutathione-Sepharose column, analyzed by SDS-PAGE, immunoblot and ROK activity as described below.

(b) Method for preparing GFP (green fluorescent protein)-tagged dominant-negative form [containing Rho-binding domain (RB)] of Rho-Kinase (ROK): The cDNA fragment encoding the dominant-negative form of ROK (2719-3237bp, containing the Rho-binding sequence (RB)] was amplified by RT-PCR using RB-specific primers linked with enzyme (Xho I and Hind III) digestion site, 5'-CGATCTCGAGGGCCTTCTGGAGGAGAGTA-3' and 5'-CGATAAGCTTCTG CATCTGAAGCTCATTCC-3'. PCR product digested with Xho I and Hind III was purified with QIAquick PCR purification Kit (Qiagen). The RB cDNA fragments were cloned into pEGFPC1 vector (Clontech) digested with Xho I and Hind III. The inserted RB sequence was confirmed by nucleotide sequencing analyses. This GFP-RB cDNA was then used for a transient expression in Met-1 cells. The GFP-RB (M.W. ≈50kDa) expressed in Met-1 cells was analyzed by SDS-PAGE, immunoblot and ROK activity assays as described below.

Cell Transfection: To establish a transient expression system, Met-1 cells were transfected with various plasmid DNAs (e.g. GFP-tagged RB cDNA or pEGFPC1 vector alone) using electroporation methods according to those procedures described previously (Chu et al., 1987). Briefly, Met-1 cells were plated at a density of 2×10^6 cells per 100 mm dish and transfected with 25 μg/dish plasmid cDNA using electroporation at 230 v and 960 μFD with a Gene Pulser (Bio-Rad). Transfected cells were grown in the culture medium for at least 24-48 h. Various transfectants were then analyzed for their protein expression (e.g. ROK-related proteins) by mmunoblot, ROK activity and cell migration assays as described below.

Microinjection Procedures: Met-1 cells or COS-7 cells (a CD44-negative cell line) (Lokeshwar, et al., 1994)

were plated onto glass coverlips and cultured in high glucose DMEM supplemented by 10% fetal bovine serum and 2mM glutamine. Both CAT and RB domains (50μg/ml) [in a microinjection buffer containing 50mM Hepes (pH 7.2), 100mM KCl, 5mM NaHPO4 (pH 7.0)] or buffer alone (as a control) was microinjected into cytosol of Met-1 cells or COS-7 using Micromanipulator 5171 and Transjector 5246 (Eppendorf, Germany). Six hours after injection, cells were fixed with 2% formaldehyde in phosphate-buffered saline for 1h and processed for immunocytochemical staining as described below.

Immunoprecipitation and Immunobloting Techniques: Met-1 cells (5 x 10^5 cells) were washed in 0.1M phosphate buffered saline (PBS; pH 7.2) and incubated with a solution containing 5 mM HEPES (pH 7.5), 150 mM KCl, 1 mM $MgCl_2$ and 100 μM GTPγS. Cells were then solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Nonidet P-40 (NP-40) buffer followed by immunoprecipitation by rabbit anti-CD44v3 (or mouse anti-RhoA antibody) plus goat anti-rabbit IgG (or goat anti-mouse IgG), respectively. The immunoprecipitated material was solubilized in SDS sample buffer and analyzed by SDS-PAGE (with 7.5% gel). Separated polypeptides were then transferred onto nitrocellulose filters. After blocking non-specific sites with 3% bovine serum albumin, the nitrocellulose filters were incubated with rabbit anti-CD44v3 antibody (5 μg/ml) or mouse anti-RhoA antibody (5 μg/ml) plus peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution). The blots were developed using ECL chemiluminescence reagent (Amersham Life Science, England) according to the manufacturer's instructions.

In some cases, NP-40 solubilized plasma membranes were incubated with sulfosuccinomidobiotin (Pierce Co., Rockford, IL) (0.1mg/ml) in labeling buffer (150μM NaCl, 0.1M HEPES, pH 8.0) for 30 min at room temperature followed by extensive dialysis against PBS buffer [0.1M phosphate buffer (pH 7.5) and 150mM NaCl]. This biotinylated material was analyzed by SDS-PAGE followed by transferring to the nitrocellulose filters and incubated with ExtrAvidin-peroxidase (Sigma Co.). After an addition of peroxidase substrate (Pierce Co.), the blots were developed using Renaissance chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

To analyze GTP binding to RhoA, the anti-RhoA-immunoprecipited materials were transferred to nitrocellulose membrane and probed with 0.25 μM [^{35}S] GTPγS (1,250Ci/mmol) in the presence or in the absence of 100 μM unlabeled GTPγS. To test the binding of RhoA to various ROK proteins, Met-1 ROK (isolated by anti-ROK-conjugated beads) (1 μg) or GST-CAT (1 μg) or GFP-RB (1 μg) were separated on an SDS-PAGE (5-12% gradient gel), transferred to nitrocellulose membrane, and probed with [^{35}S]GTPγS•GST-RhoA as described previously (Amano et al., 1997). The radioactively labeled bands were detected by fluorography.

In some cases, GST-tagged CAT fusion protein or cell lysate of Met-1 cells [transiently transfected with either GFP-tagged RB ROK cDNA or pEGFPC1 vector alone] were immunoblotted with mouse anti-GST (5 μg/ml) or mouse anti-GFP antibody (5 μg/ml) for 1h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

The procedures for [^{32}P]ADP-ribosylation with botulinum toxin C3 were the same as described previously (Aktories et al., 1987; Ohashi and Narumiya, 1987). Radioactively labeled proteins were immunoprecipitated by anti-CD44v3 antibody followed by SDS-PAGE and autoradiographic analyses.

GTPase Activity Assay: The GTPase activity was performed as described previously with the following modification (Kabcenell et al., 1990; O'Neil et al., 1990). The $CD44v_{3,8\text{-}10}$-bound RhoA complex (20 pmole) (with or without C3-mediated ADP-ribosylation reaction) were incubated in the GTPase assay buffer [20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.1% cholate, and 1 mM dithiothreitol (DTT) in the presence of 1 μM [γ-^{32}P]GTP (4 x 10^4 cpm/pmole) and 0.1 mM ATP] in a reaction volume of 50 μl at 4°C for 30min. The samples were then incubated at 37°C for various time intervals. Following incubation, 100 μl of 1% BSA, 0.1% dextran sulfate made in 20mM phosphate buffer (pH 8.0) was added to the reaction mixtures followed by the addition of 750 μl of activated charcoal suspension containing 20mM phosphate buffer (pH 8.0). Following incubation at 4°C for 30 min, the reaction mixtures were centrifuged, and $^{32}P_i$ released in the supernatant was determined by liquid scintillation counting. The results are expressed as pmole of Pi released per μg of protein. In control samples,

the non-specific release of P_i caused by the background level of GTPase activity (associated with preimmune rabbit IgG-bead associated materials) was determined. The non-specific release of P_i in control samples was less than 10% of that released by CD44v3-bound RhoA complex samples and has been subtracted. Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically by Student's t test and statistical significance was set at $p<0.01$.

Double Immunofluorescence Staining: Met-1 cells [microinjected with CAT or RB] grown in the absence or presence of certain agents [e.g. rabbit anti-CD44v3 antibody (50μg/ml) or preimmune rabbit serum (50μg/ml) or cytochalasin D (20μg/ml)] were first washed with PBS [0.1M phosphate buffer (pH 7.5) and 150mM NaCl] buffer and fixed by 2% paraformaldehyde. Subsequently, cells were surface stained with FITC-labeled rat anti-CD44 antibody. These FITC-labeled cells were then rendered permeable by ethanol treatment followed by incubating with Rhodamine (Rh)-conjugated mouse anti-Ank1 or rabbit anti-Ank3 IgG. To detect non-specific antibody binding, FITC-CD44 labeled cells were incubated with Rh-conjugated normal mouse IgG. No labeling was observed in such control samples. The fluorescein- and rhodamine-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA).

Protein Phosphorylation Assay: The kinase reaction was carried out in 50 μl of the reaction mixture containing 40 mM Tris-HCl (pH7.5), 2 mM EDTA, 1 mM DTT, 7 mM $MgCl_2$, 0.1% CHAPS, 0.1 μM calyculin A, 100 μM [γ-^{32}P]ATP (15-600 mCi/mmol), purified enzymes (e.g. 100 ng Met-1 ROK, or 20 ng GST-CAT) and 1 μg cellular proteins (e.g. smooth muscle myosin light chain, $CD44v_{3,8-10}$ and FLAG-tagged CD44 cytoplasmic domain (FLAG-CD44cyt)] in the presence or absence of GTPγS•GST-RhoA (complexed with $CD44v_{3,8-10}$) (1 μM) or GTPγS•RhoA (dissociated from $CD44v_{3,8-10}$-RhoA complex by 0.6 M NaCl) (1 μM) or GTPγS•GST-RhoA fusion protein (1 μM) (or GST-RhoA alone) or inhibitors such as GFP-RB (1 μM) or staurosporine (1μM). After an incubation for various time intervals (e.g. 0, 10, 20, 30, 60 and 120 min) at 30°C, the reaction mixtures were boiled in SDS-sample buffer and subjected to SDS-PAGE. The protein bands were revealed by silver stain and the radiolabeled bands were visualized by fluorography or analyzed by liquid scintillation counting as described previously (Matsui, et al., 1998).

Binding of ^{125}I-labeled Ankyrin To $CD44v_{3,8-10}$ and FLAG-Tagged CD44 Cytoplasmic Domain (FLAG-CD44cyt): Purified ^{125}I-labeled ankyrin (≈0.32 nM protein, 1.5 x 10^4 cpm/ng) was incubated with purified $CD44v_{3,8-10}$ [bound to anti-CD44v3-conjugated beads] or FLAG-tagged CD44 cytoplasmic domain (FLAG-CD44cyt) [bound to anti-FLAG-conjugated beads] (≈0.75 μg protein in phosphorylated or unphosphorylated form) in 0.5 ml of the binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% (w/v) BSA and 0.05% Triton X-100]. Binding was carried out at 4°C for 5 h under equilibrium conditions. Equilibrium conditions were determined by performing a time course (e.g. 1 h-10 h) of the binding reaction. Following binding, the beads were washed in the binding buffer and the bead bound radioactivity was determined. Non-specific binding was determined in the presence of either a 100 fold excess of unlabeled ankyrin or using bovine serum albumin conjugated Sepharose beads. Non-specific binding was approximately 20-30% of the total binding, and was subtracted from the total binding.

Tumor Cell Migration Assays: Twenty-four transwell units were used for monitoring in vitro cell migration as described previously (Merzak et al., 1994). Specifically, the 8 μm porosity polycarbonate filters were used for the cell migration assay (Merzak et al., 1994). Met-1 cells [≈1 x 10^4 cells/well in phosphate buffered saline (PBS), pH 7.2] [in the presence or absence of rabbit anti-CD44v3 antibody (50 μg/ml) or cytochalasin D (20μg/ml)] were placed in the upper chamber of the transwell unit. In some cases, Met-1 cells were transfected with either GFP-tagged RB cDNA or pEGFPC1 vector alone. The growth medium containing high glucose DMEM supplemented by 10% fetal bovine serum was placed in the lower chamber of the transwell unit. After 18 h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (Merzak et al., 1994). Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically using the Student's t test and statistical significance was set at $p<0.01$.

RESULTS

Characterization of CD44 Variant Isoform(s) in Met-1 Cells

Breast tumor cells (Met-1 cell line) were derived from a high metastatic potential tumor in transgenic mice expressing polyoma virus middle T oncogene (Cheung et al., 1997; Guy et al., 1992). They are capable of inducing a high level of intratumoral microvessel formation (Cheung et al., 1997). Our recent results indicate that Met-1 cells express a CD44 variant isoform, $CD44v_{3,8\text{-}10}$ which contains v3 and v8-10 exon insertions (Bourguignon et al., 1998). This $CD44v_{3,8\text{-}10}$ variant exon structure was previously identified in human breast carcinoma samples (Bourguignon et al., 1998; Iida and Bourguignon, 1995). In this study we have used biotinylation labeling of NP-40 solubilized plasma membranes and SDS-PAGE analyses to resolve a number of cellular proteins (ranging from ≈300kDa to ≈25kDa) (Fig. 1, lane 1) in the membrane-cytoskeleton fraction of Met-1 cells. Immunoblotting with anti-CD44v3 antibody indicates that a single polypeptide (M. W. ≈ 260kDa) expressed in Met-1 cells belongs to the $CD44v_{3,8\text{-}10}$ isoform (Fig. 1, lane 3). No $CD44v_3$-containing material is observed in control samples when preimmune rabbit serum is used (Fig. 1, lane 2). These results are consistent with a previous report which determined that the molecular mass of this $CD44v_{3,8\text{-}10}$ isoform to be ≈260kDa (Bennet et al., 1995).

Recently, the 260kDa $CD44v_{3,8\text{-}10}$ molecule has been found to be closely associated with a matrix metalloproteinase (MMP-9) and also interacts with the cytoskeleton during "invadopodia" formation and tumor cell migration (Bourguignon et al., 1998). These findings suggest that $CD44v_{3,8\text{-}10}$ and the associated cytoskeleton play an important role in metastatic tumor cell behavior (Bourguignon et al., 1998). The question regarding which transmembrane signaling pathways are involved in regulating these $CD44v_{3,8\text{-}10}$-mediated cytoskeletal activities in metastatic breast tumor cells is the focus of this study.

Physical Linkage Between $CD44v_{3,8\text{-}10}$ and RhoA GTPase

Certain cytoskeleton functions are regulated by activation of Rho GTPases such as RhoA, Rac1 and Cdc42 (Hall, 1998; Narumiya, 1996). Specifically, RhoA is required for actin filament bundling to regulate stress fiber formation and acto-myosin-based contractility (Hall, 1998; Narumiya, 1996). The rationale for our focusing on the interaction between small molecular weight GTPases and $CD44v_{3,8\text{-}10}$-cytoskeleton-linked invasive phenotypes is based on a previous report by Tsukita and co-workers suggesting the involvement of CD44-associated cytoskeletal proteins (ERM) in Rho-induced cytoskeletal effects (Hirao et al., 1996). In this study we have detected that the 260kDa $CD44v_{3,8\text{-}10}$ band and a 25 kDa RhoA-like protein are physically associated as a complex (Fig. 1, lane 4 and lane 5). Specifically, we have used Met-1 cells and anti-RhoA-mediated or anti-CD44v3-immunprecipitation followed by anti-CD44v3 immunoblot (Fig. 1, lane 4) or anti-RhoA immunoblot (Fig. 1, lane 5), respectively. Together, our results clearly indicate that the $CD44v_{3,8\text{-}10}$ band is revealed in anti-RhoA-immunoprecipitated materials (Fig. 1, lane 4). The RhoA band is also detected in the anti-CD44v3-immunoprecipitated materials (Fig. 1, lane 5). Control results confirm the specificity of these immunological techniques. For example, very little RhoA is detected in anti-CD44v3-immunprecipitated materials blotted by an anti-RhoA-free serum (anti-RhoA antibody pre-absorbed by an excess amount of RhoA) (Fig. 1, lane 6). Similarly, no $CD44v_{3,8\text{-}10}$ is observed in anti-RhoA-immunprecipitated materials blotted by an anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of $CD44v_{3,8\text{-}10}$) (data not shown). Preliminary data indicate that approximately 50% of CD44 is associated with RhoA in the plasma membrane fraction of Met-1 cells. These findings establish the fact that $CD44v_{3,8\text{-}10}$ and RhoA are closely associated with each other as a complex in vivo.

Using an in vitro [^{35}S]GTPγS binding assay, we have determined that the 25 kDa RhoA-like protein displays guanine nucleotide binding activity (Fig. 1, lane 7). In the presence of excess amounts of unlabeled GTPγS, no radioactive labeling was observed (Fig. 1, lane 8). Since RhoA has been shown to possess an intrinsic GTPase activity (Hall, 1998), we decided to test whether there is GTPase activity associated with this $CD44v_{3,8\text{-}10}$-RhoA complex. As shown in Fig. 2, the $CD44v_{3,8\text{-}10}$-RhoA complex clearly displays GTPase activity which hydrolyzes [γ-^{32}P]GTP in a linear time-dependent manner (Fig. 2A).

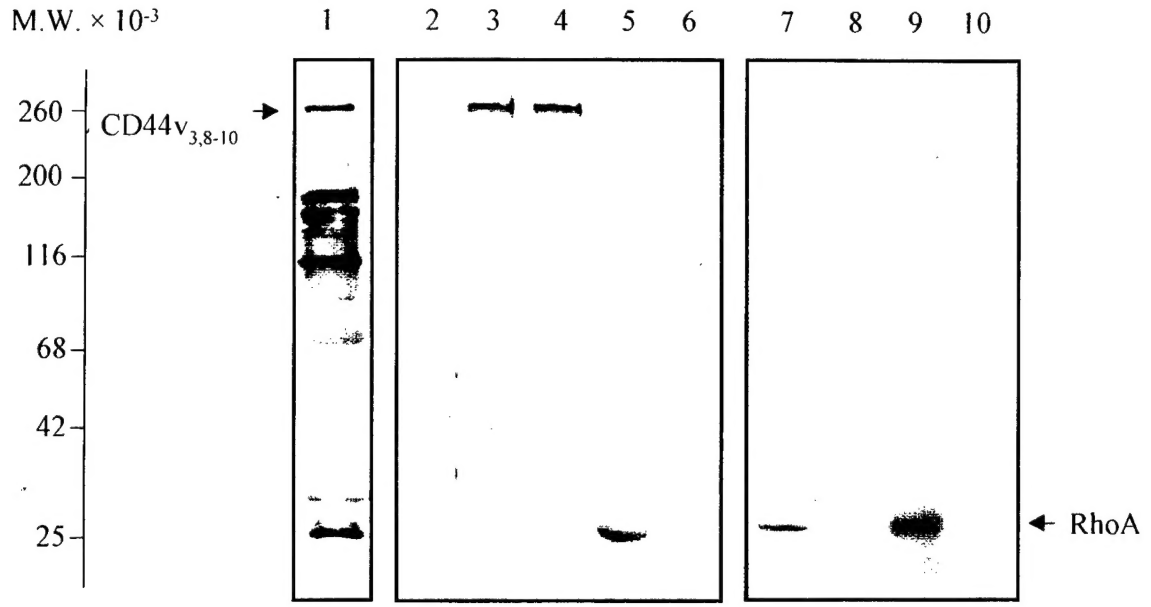

Fig. 1: Analysis of $CD44v_{3,8-10}$ expression and $CD44v_{3,8-10}$-RhoA complex in mouse breast tumor cell (Met-1 cells).

Met-1 cells (5×10^5 cells) were washed in 0.1M phosphate buffered saline (PBS; pH 7.2) and incubated with a solution containing 5 mM HEPES (pH 7.5), 150 mM KCl, 1 mM $MgCl_2$, and 100 µM GTPγS. Cells were then solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 1% Nonidet P-40 (NP-40) buffer followed by immunoblot and/or immunoprecipitation by rabbit anti-CD44v3 or mouse anti-RhoA. The immunoprecipitated material was solubilized in SDS sample buffer and analyzed by SDS-PAGE (with 7.5% gel). The procedures for identifying RhoA by [^{35}S]GTPγS binding or botulinum toxin C3-mediated [^{32}P]ADP-ribosylation were described in the Materials and Methods.

Lane 1: Biotinylated total NP-40 solubilized plasma membrane-associated proteins.

Lane 2: Immunoblot of Met-1 cells with preimmune rabbit serum.

Lane 3: Identification of $CD44v_{3,8-10}$ by immunoblotting Met-1 cells with rabbit anti-$CD44v_3$-specific antibody.

Lane 4: Detection of $CD44v_{3,8-10}$ in the complex by anti-RhoA-mediated immunoprecipitation followed by immunoblotting with anti-$CD44v_3$-specific antibody.

Lane 5: Detection of RhoA in the complex by anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with anti-RhoA-specific antibody.

Lane 6: Control experiments to detect RhoA in the complex by anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with anti-RhoA-free serum (anti-RhoA antibody pre-absorbed by an excess amount of RhoA).

Lane 7: Autoradiogram of [^{35}S]GTPγS binding (in the absence of unlabeled GTPγS) to RhoA obtained from anti-RhoA-mediated immunoprecipited materials.

Lane 8: Autoradiogram of [^{35}S]GTPγS binding (in the presence of unlabeled GTPγS) to RhoA obtained from anti-RhoA-mediated immunoprecipited materials.

Lane 9: Autoradiogram of [^{32}P]ADP-ribosylation of RhoA obtained from anti-RhoA-mediated immunoprecipited materials in the presence of botulinum toxin C3.

Lane 10: Autoradiogram of [^{32}P]ADP-ribosylation of RhoA obtained from anti-RhoA-mediated immunoprecipited materials in the absence of botulinum toxin C3.

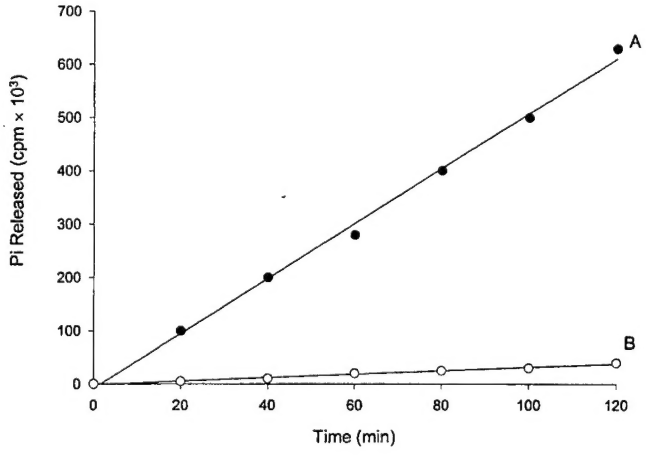

Fig. 2: GTPase activity assay of RhoA complexed with $CD44v_{3,8-10}$

Aliquots of RhoA complexed with $CD44v_{3,8-10}$ (as described in Fig. 1) (20 pmole) were incubated with 1 µM [γ-^{32}P]GTP (4×10^4 cpm/pmole) for various time periods. The amount of $^{32}P_i$ liberated was determined as described under Materials and Methods. Data represent an average of triplicates. The standard deviation was less than 5%. (A: in the absence of C3-mediated ADP-ribosylation; B: in the presence of C3-mediated ADP-ribosylation).

However, it is also possible that other small GTP-binding proteins, such as Rac or Cdc42, are also complexed with $CD44v_{3,8-10}$ and contribute some of the GTPase activity measured in these experiments. One way to address this issue is to utilize specific Rho inhibitors. For example, RhoA (but not Rac and Cdc42) is a substrate for certain bacterial toxins such as Clostridium botulinum C3 toxin (Aktories et al., 1987; Ohashi and Narumiya, 1987). C3 toxin ADP-ribosylates RhoA (but not Rac and Cdc42) and inactivates RhoA GTPase (Aktories et al., 1987; Ohashi and Narumiya, 1987). Here, we have found that the 25 kDa protein complexed with $CD44v_{3,8-10}$ can be [^{32}P]ADP-ribosylated by C3 toxin (Fig. 1, lane 9). In a control sample, when [^{32}P]ADP-ribosylation of $CD44v_{3,8-10}$ bound RhoA was carried out in the absence of C3 toxin, no labeling of RhoA was observed (Fig. 1, lane 10). Most importantly, C3-mediated ADP-ribosylation of RhoA complexed with $CD44v_{3,8-10}$ eliminates more than 90% of the GTPase activity (Fig. 2B). These data suggest that $CD44v_{3,8-10}$-bound RhoA displays GTPase activity and also contains a site for ADP-ribosylation mediated by the RhoA inhibitor.

Rho-Kinase (ROK) As A Downstream Effector for RhoA (Complexed with $CD44v_{3,8-10}$) in Met-1 Cells

(A) Characterization of Rho-Kinase (ROK) In Met-1 Cells:

In order to identify the downstream target(s) for RhoA GTPase complexed with $CD44v_{3,8-10}$ (Fig. 1), we have initially focused on Rho-Kinase (ROK), one of the known effectors for Rho GTPases (Leung et al., 1996; Matsui et al., 1996). ROK is composed of four functional domains: a kinase domain (catalytic site or CAT), a coiled-coil domain, a Rho-binding (RB) domain and a pleckstrin-homology (PH) domain (Leung et al., 1996; Matsui et al., 1996) (Fig. 3A-a). Both the kinase (CAT) and Rho-binding (RB) domains share a great deal of sequence homology with a family of related kinases known to bind Rho GTPase and participate in cell motility and cytoskeleton functions (Amano et al., 1997). To analyze the expression of ROK in Met-1 cells, we have prepared a ROK-specific antibody which is raised against the N-terminal sequence of ROK. Using the ROK-specific antibody and immunoblot of cellular proteins from Met-1 cells, we have identified a 160 kDa polypeptide (Fig. 3B, lane 1). We believe that this antibody is specific since no protein is detected in these cells in the presence of preimmune rabbit IgG (Fig. 3B, lane 4). Furthermore, we have incubated [^{35}S]GTPγS•GST-RhoA with nitrocellulose papers containing ROK [prepared by anti-ROK-mediated immunoprecipitation followed by transfer to nitrocellose papers]. Our results indicate that the 160 kDa polypeptide is capable of binding to GST-RhoA directly (Fig. 3B, lane 2), (but not GST alone) (Fig. 3B, lane 3), and therefore, it is a RhoA-binding protein.

In addition, we have constructed two ROK cDNAs (e.g. CAT cDNA and RB cDNA). These two constructs encode for proteins consisting of 537 amino acids [1-1611 bp (or aa1-aa537), designated as the constitutively active form of ROK containing the catalytic domain (CAT, also the kinase domain)] (Fig. 3A-b) and 173 amino acids [2719-3237bp (or aa907-aa1070), designated as the dominant-negative form of ROK containing the Rho-binding domain (RB)] (Fig. 3A-c) , respectively (Amano et al., 1997). The CAT cDNA was cloned into a GST-tagged expression vector (pESP-2 vector) and then introduced to an eukaryotic expression system such as yeast (S. pombe) to produce GST-CAT fusion protein. The RB cDNA was cloned into a green fluorescent protein (GFP)-tagged expression vector (pEFGPC1 vector) followed by a transient transfection of GFP-RB cDNA into Met-1 cells.

Using mouse anti-GST (Fig. 3C, lane 1) and rabbit anti-ROK immunoblot (Fig. 3C, lane 2) analyses, we have detected the GST-CAT fusion protein as a single polypeptide with a molecular mass of 80 kDa. We have also identified the presence of GFP-RB (M.W. ≈50kDa) in Met-1 cells (Fig. 3D, lane 1) by anti-GFP immunoblot. No immuno-labeling was observed if normal mouse IgG was used in these samples (Fig. 3C, lane 4 and Fig. 3D, lane 4). To test the ability of these ROK-related proteins to bind RhoA, we have incubated nitrocellulose papers containing ROK-related proteins [e.g. GST-CAT or GFP-RB] with [^{35}S]GTPγS•GST-RhoA. Our results indicate that GST-CAT fails to bind RhoA (Fig. 3C, lane 3); whereas GFP-RB binds specifically to [^{35}S]GTPγS•GST-RhoA (Fig. 3D, lane 2) [but not GST alone] (Fig. 3D, lane 3). These findings strongly suggest that the Rho-binding site reside in the RB domain but not in the CAT domain.

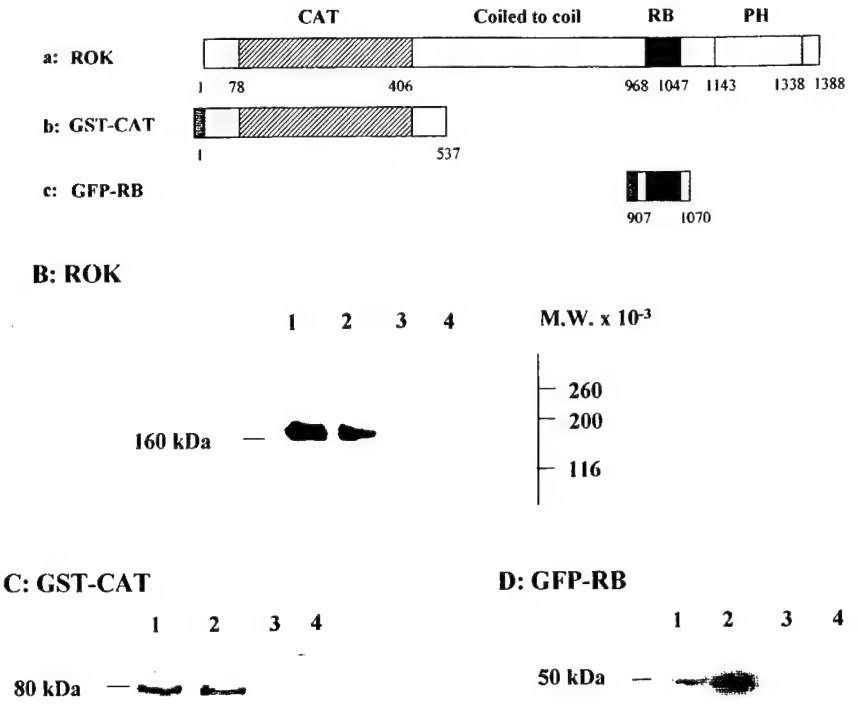

Fig. 3: Characterization of three ROK-related proteins (e.g. 160 kDa protein, GFP-CAT and GFP-RB).

Three ROK-related proteins (e.g. 160 kDa protein, GFP-CAT and GFP-RB) were analyzed by immunoblot analyses and [^{35}S]GTPγS•GST-RhoA binding as described in the Materials and Methods.

A: Illustration of ROK-related mutant cDNA constructs.

a: The full-length ROK contains a kinase domain (catalytic domain or CAT), a coiled-coil domain, a rho-binding (RB) domain and a pleckstrin-homology (PH) domain.

b: GST-CAT fusion protein represents the catalytic (CAT) domain of ROK (aa1-aa537).

c:GFP-RB fusion protein represents the rho-binding (RB) domain (aa907-aa1070).

B: ROK expression in Met-1 cells.

Lane 1: Identification of ROK by immunoblotting Met-1 cells with rabbit anti-ROK-specific antibody.

Lane 2: Autoradiogram of [^{35}S]GTPγS•GST-RhoA binding to ROK (obtained from anti-ROK-mediated immunoprecipited materials).

Lane 3: Autoradiogram of [^{35}S]GTPγS binding [in the presence of GST alone (without RhoA)] to ROK (obtained from anti-ROK-mediated immunoprecipited materials).

Lane 4: Immunoblot of Met-1 cells with preimmune rabbit serum.

C: Characterization of GST-CAT fusion protein.

Lane 1: Immunoblot of GST-CAT with mouse anti-GST antibody.

Lane 2: Immunoblot of GST-CAT with rabbit anti-ROK antibody.

Lane 3: Autoradiogram of [^{35}S]GTPγS•GST-RhoA binding to GST-CAT.

Lane 4: Immunoblot of GST-CAT with normal mouse IgG.

D: GFP-RB expression in Met-1 cells transfected with GFP-RB cDNA.

Lane 1: Immunoblot of Met-1 transfectants with mouse anti-GFP antibody.

Lane 2: Autoradiogram of [^{35}S]GTPγS•GST-RhoA binding to GFP-RB (obtained from Met-1 transfectants using anti-GFP-mediated immunoprecipition).

Lane 3: Autoradiogram of [^{35}S]GTPγS binding [in the presence of GST alone (without RhoA)] to GFP-RB (obtained from Met-1 transfectants using anti-GFP-mediated immunoprecipited materials).

Lane 4: Immunoblot of Met-1 transfectants with normal mouse IgG.

To determine whether there is kinase(s) activity associated with the 160 kDa protein and/or the two ROK-related proteins (e.g. GST- CAT or GFP-RB), we have carried out the kinase reaction using smooth muscle myosin light chain as a substrate (Amano et al., 1997; Leung et al., 1996) in the presence of various ROK-related proteins including 160 kDa (isolated from Met-1 cells by anti-ROK immuno-beads), GST-CAT and GFP-RB] and [γ-^{32}P]ATP. As shown in Fig. 4A, a significant amount of 160 kDa protein-mediated myosin light chain (MLC) phosphorylation occurs in the presence of GTPγS•GST-RhoA (Fig. 4A, lane 1). GST-CAT is also found to phosphorylate myosin light chain in the absence of GTPγS•GST-RhoA (Fig. 4A, lane 2). A minimal amount of

myosin light chain phosphorylation is detected in the presence of GFP-RB plus GTPγS•GST-RhoA (Fig. 4A, lane 3) or staurosporine (a serine-threonine kinase inhibitor)-treated 160kDa ROK in the presence of GTPγS•GST-RhoA (Fig. 4A, lane 4). These results are consistent with previous findings (Amano et al., 1996; Amano et al., 1997) suggesting this 160 kDa polypeptide expressed in Met-1 cells belongs to a family of kinases known to bind RhoA GTPase [also called Rho-Kinase (ROK)] and to phosphorylate MLC. The CAT (but not RB) of ROK acts as a constitutively active form of kinase.

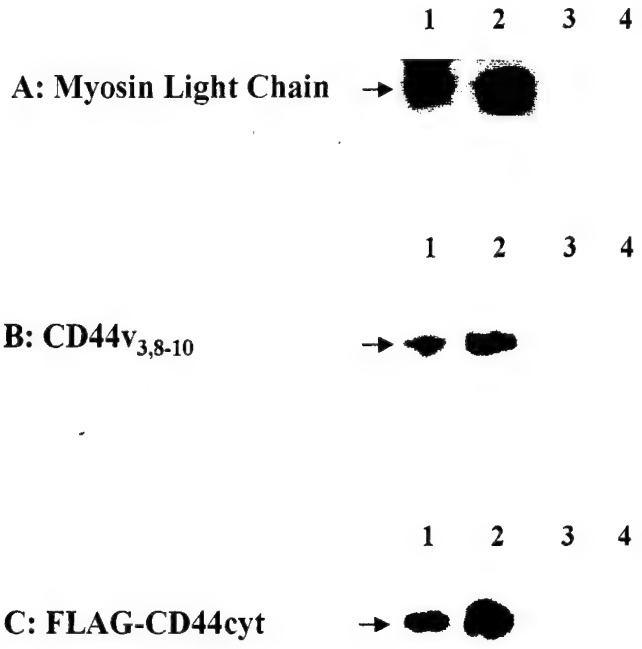

Fig. 4: *Effects of ROK and ROK mutant proteins on protein phosphorylation.*

The kinase reaction was carried out in the reaction mixture containing 100 μM [γ-^{32}P]ATP (15-600 mCi/mmol), purified enzymes [e.g. 100 ng Met-1 ROK (isolated from anti-ROK-conjugated beads) or 20 ng GST-CAT] and 1 μg cellular proteins [e.g. smooth muscle myosin light chain, CD44v$_{3,8-10}$ and FLAG-tagged CD44 cytoplasmic domain (FLAG-CD44cyt)] with or without GTPγS•RhoA (isolated from CD44v$_{3,8-10}$-RhoA complex) (1 μM) or GTPγS•GST-RhoA (1 μM) or GST-RhoA (1 μM) or certain inhibitor (e.g. GFP-RB) as described in the Materials and Methods.

A: Myosin light chain phosphorylation.

Lane 1: Autoradiogram of myosin light chain phosphorylation by 160 kDa ROK (isolated from Met-1 cells) activated by GTPγS•GST-RhoA.

Lane 2: Autoradiogram of myosin light chain phosphorylation by GST-CAT in the absence of GTPγS•GST-RhoA.

Lane 3: Autoradiogram of myosin light chain phosphorylation by GFP-RB (isolated from Met-1 transfectants) in the presence of GTPγS•GST-RhoA.

Lane 4: Autoradiogram of myosin light chain phosphorylation by 160kDa ROK in the presence of unactivated GST-RhoA.

B: CD44v$_{3,8-10}$ phosphorylation.

Lane 1: Autoradiogram of CD44v$_{3,8-10}$ phosphorylation by 160 kDa ROK (isolated from Met-1 cells) in the presence of GTPγS•RhoA (isolated from CD44v$_{3,8-10}$-RhoA complex).

Lane 2: Autoradiogram of CD44v$_{3,8-10}$ phosphorylation by GST-CAT in the absence of GTPγS•RhoA.

Lane 3: Autoradiogram of CD44v$_{3,8-10}$ phosphorylation by 160 kDa ROK in the presence of GTPγS•RhoA (isolated from CD44v$_{3,8-10}$-RhoA complex) and GFP-RB.

Lane 4:Autoradiogram of CD44v$_{3,8-10}$ phosphorylation by 160 kDa ROK in the presence of unactivated GST-RhoA.

C: FLAG-tagged CD44cyt phosphorylation.

Lane 1: Autoradiogram of FLAG-CD44cyt phosphorylation by GTPγS•GST-RhoA-activated ROK.

Lane 2: Autoradiogram of FLAG-CD44cyt phosphorylation by GST-CAT in the absence of GTPγS•GST-RhoA.

Lane 3: Autoradiogram of FLAG-CD44cyt phosphorylation by 160kDa ROK in the presence of GTPγS•RhoA (isolated from CD44v$_{3,8-10}$-RhoA complex) and GFP-RB.

Lane 4: Autoradiogram of FLAG-CD44cyt phosphorylation by 160kDa ROK in the presence of unactivated GST-RhoA.

(B) Effects of ROK-Mediated CD44v$_{3,8-10}$ Phosphorylation On Membrane-Cytoskeleton Interaction And Tumor Cell Migration :

To identify other possible cellular substrate(s) of ROK in Met-1 cells, we have examined the ability of various ROK-related proteins (e.g. ROK, GST-CAT and GFP-RB) to CD44 [e.g. CD44v$_{3,8-10}$ and/or E. coli-derived FLAG tagged-CD44 cytoplasmic domain fusion protein (designated as FLAG-CD44cyt)]. Specifically, we have found that GTPγS•RhoA stimulates 160 kDa ROK to phosphorylate CD44v$_{3,8-10}$ (Fig. 4B, lane 1). In the presence of unactivated RhoA (e.g. GST-RhoA), ROK-mediated CD44v$_{3,8-10}$ phosphorylation is significantly blocked (Fig. 4B, lane 4). The constitutively active form of ROK (GST-CAT) also causes phosphorylation of CD44v$_{3,8-10}$ (Fig. 4B, lane 2); whereas the dominant-negative form (GFP-RB) of ROK inhibits ROK-mediated CD44v$_{3,8-10}$ phosphorylation in the presence of GTPγS•RhoA (Fig. 4B, lane 3).

Similarly, our data show that the 160 kDa protein isolated from Met-1 cells is capable of phosphorylating the cytoplasmic domain of CD44 (FLAG-CD44cyt) (Fig. 4C). Specifically, there is a significant increase of 160kDa protein-mediated phosphorylation of FLAG-CD44cyt in the presence of activated GTPγS•GST-RhoA

(Fig. 4C, lane 1). The level of ROK-mediated FLAG-CD44cyt phosphorylation becomes very low if unactivated GST-RhoA is used (Fig. 4C, lane 4). Furthermore, GST-CAT (Fig. 4C, lane 2) causes a significant stimulation of FLAG-CD44cyt phosphorylation (with no addition of GTPγS•GST-RhoA). In the presence of GFP-RB, phosphorylation of FLAG-CD44cyt by GTPγS•GST-RhoA-activated ROK is inhibited. These findings support the notion that GST-CAT acts as a constitutively active form of ROK and GFP-RB functions as a dominant-negative form of ROK. Together, we conclude that the cytoplasmic domain of CD44 serves as one of the cellular substrates for the 160 kDa Rho-binding-dependent kinase, such as ROK.

In addition, we have analyzed the stoichiometry of CD44 phosphorylation by Rho-Kinase (ROK), along with a time course, and myosin light chain phosphorylation as a positive control. As shown in Fig. 5, ≈1.25mol of phosphate is maximally incorporated into 1 mol of FLAG-CD44cyt (the cytoplasmic domain of CD44 fusion protein tagged with FLAG) by ROK in the presence of activated GTPγS•GST-RhoA in a time-dependent manner (Fig. 5A-a). Phosphorylation of CD44cyt appears to be minimal (at most ≈0.1mole of phosphate incorporated into per mol of FLAG-CD44cyt) using ROK treated with unactivated GST-RhoA (Fig. 5A-b). In addition, we have found that approximately 1 mol of phosphate becomes maximally incorporated into 1 mol of myosin light chain (MLC) by GTPγS•GST-RhoA-activated ROK (Fig. 5B-a). In contrast, the level of ROK-mediated MLC phosphorylation is greatly reduced [only ≈0.28 mol of phosphate incorporated into 1 mol of myosin light chain (MLC)] in the presence of unactivated GST-RhoA (Fig. 5B-b). Since the stoichiometry of CD44 phosphorylation by activated ROK is comparable to that of MLC phosphorylation (by activated ROK), we conclude that CD44 is a good cellular substrate for ROK.

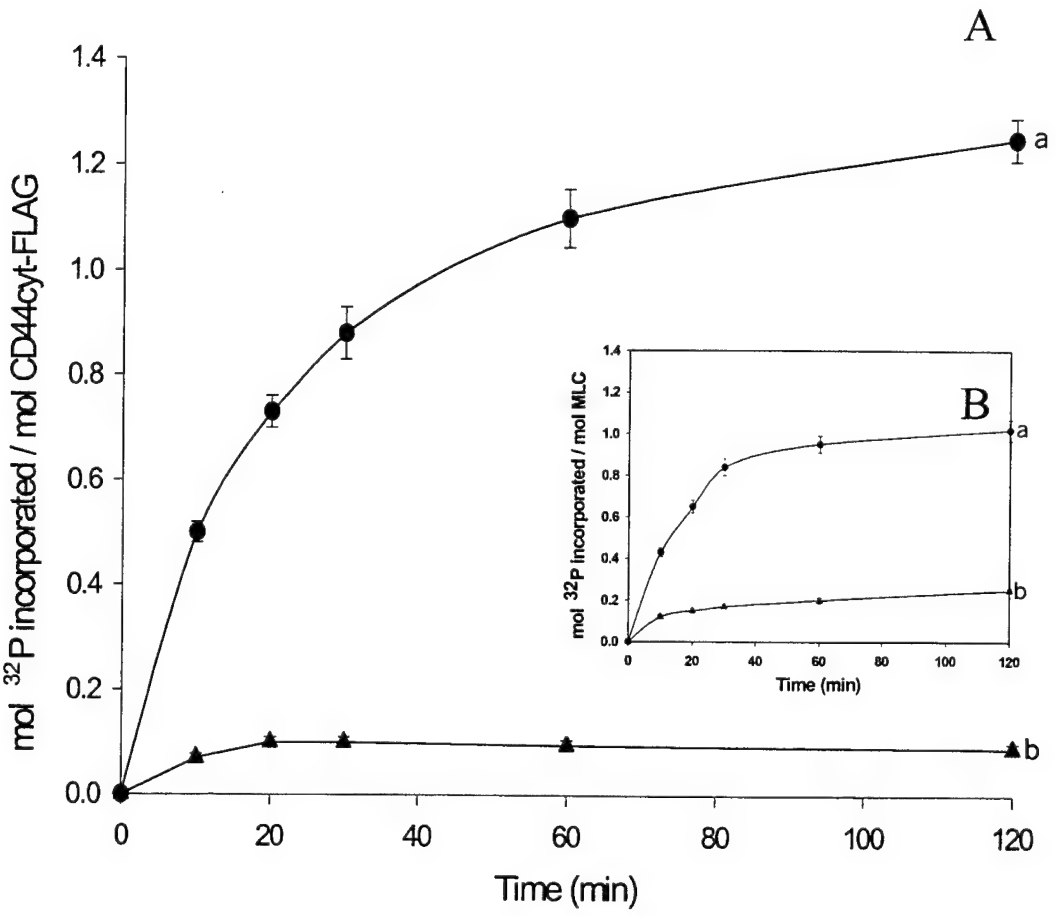

Fig 5: ***Measurement of FLAG-CD44cyt phosphorylation by ROK-related proteins.***

The kinase reaction used in these experiments was the same as described in the legend of Fig. 4. The amount of [γ-^{32}P]ATP incorporated into FLAG-CD44cyt by 160kDa ROK (in the presence of activated GTPγS•GST-RhoA or unactivated GST-RhoA) was measured at various time intervals (e.g. 0, 10, 20, 30, 60 and 120 min) as described in the Materials and Methods. Data are means ±SEM of triplicate determinations from 3-5 different experiments.

A-a: ROK-mediated FLAG-CD44cyt phosphorylation in the presence of activated GTPγS•GST-RhoA.

A-b: ROK-mediated FLAG-CD44cyt phosphorylation in the presence of unactivated GST-RhoA.

B-a: ROK-mediated myosin light chain (MLC) phosphorylation in the presence of activated GTPγS•GST-RhoA.

B-b: ROK-mediated mysoin light chain (MLC) phosphorylation in the presence of unactivated GST-RhoA.

Phosphorylation of CD44's cytoplasmic domain has been shown to be important for its interaction with the cytoskeletal proteins such as ankyrin (Bourguignon et al., 1992; Kalomiris and Bourguignon, 1989). In this study we decided to examine the effect of ROK-mediated CD44 phosphorylation on ankyrin binding. Specifically, the highly phosphorylated form of FLAG-CD44cyt [by GTPγS•GST-RhoA-activated ROK] (as shown in Fig. 4, lane 1) or the minimally phosphorylated form of FLAG-CD44cyt [using ROK treated with unactivated GST-RhoA (as shown in Fig. 4, lane 4)] was incubated with various concentrations of ^{125}I-labeled ankyrin under equilibrium binding conditions. Our results indicate that the total amount of ^{125}I-ankyrin binding to the highly phosphorylated form of FLAG-CD44cyt is significantly higher (Fig. 6C-a) than that detected in the minimally phosphorylated form of FLAG-CD44cyt (Fig. 6C-b). Further Scatchard plot analyses indicate that ankyrin binds to either highly phosphorylated FLAG-CD44cyt or minimally phosphorylated CD44cyt at a single site (Fig. 6A and B). Importantly, the highly phosphorylated FLAG-CD44cyt displays at least 40-fold higher ankyrin binding affinity (with an apparent $K_d \approx 0.05$nM) (Fig. 6A) than the minimally phosphorylated FLAG-CD44cyt (with an apparent $K_d \approx 2.1$nM) (Fig. 6B). These results clearly support the notion that phosphorylation of the cytoplasmic domain of CD44 by ROK enhances its binding interaction with ankyrin which may be required for cytoskeleton-mediated function.

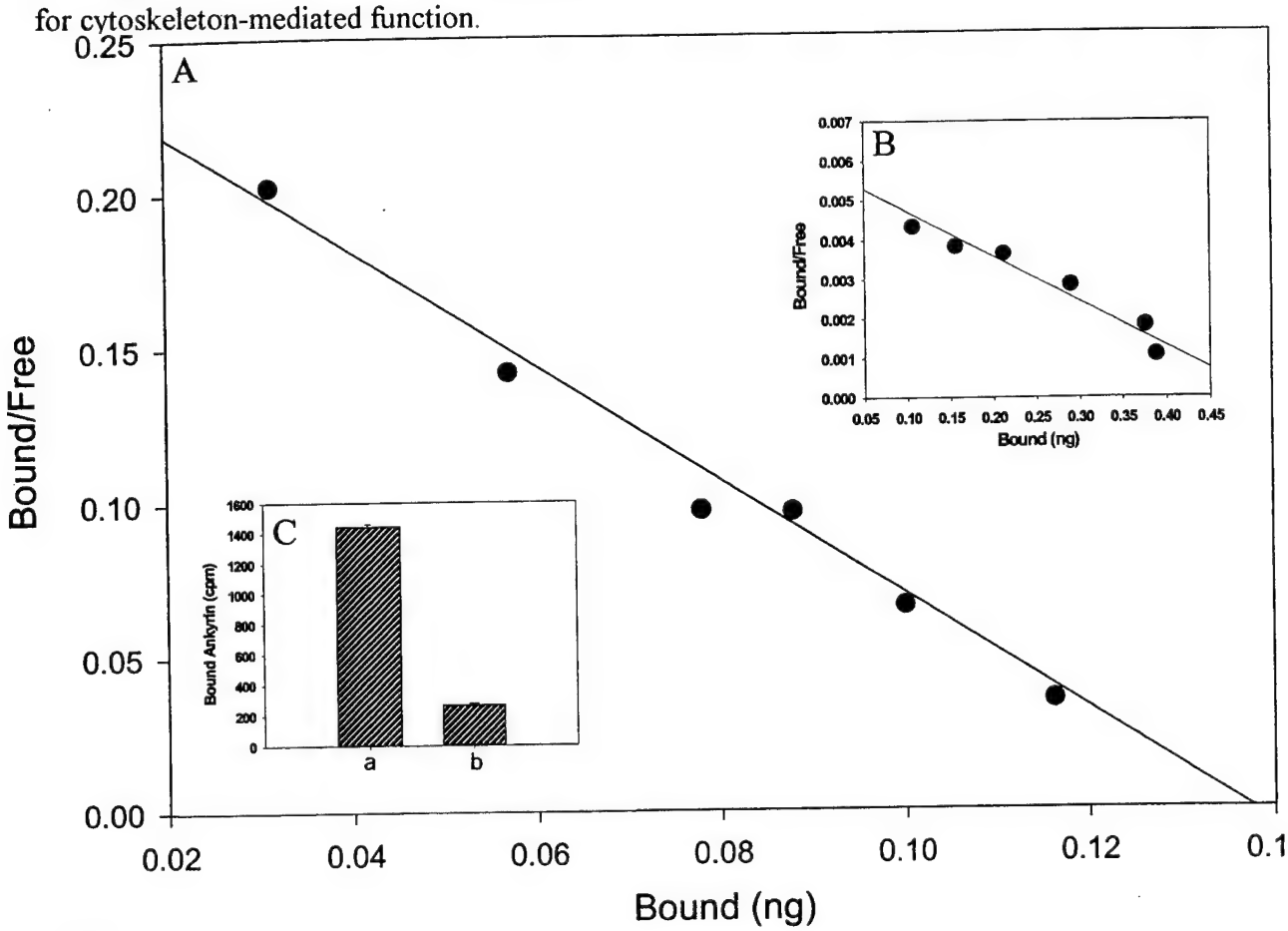

Fig. 6: ***^{125}I-Ankyrin binding to ROK-phosphorylated FLAG-CD44cyt by Scatchard plot analyes.***

Purified ^{125}I-labeled ankyrin (≈0.32 nM protein, 1.5 x 10^4 cpm/ng) was incubated with FLAG-tagged CD44 cytoplasmic tail (FLAG-CD44cyt) [bound to anti-FLAG-conjugated beads] (≈0.75μg protein in ROK-phosphorylated or unphosphorylated form) in 0.5 ml of the binding buffer [20 mM Tris-HCl (pH 7.4), 150 mMNaCl, 0.1% (w/v) BSA and 0.05% Triton X-100] as described in the Materials and Methods. Following binding, the beads were washed in the binding buffer and the bead bound radioactivity was determined. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled ankyrin. A: Scachard plot analysis of the ^{125}I-Ankyrin binding to highly phosphorylated FLAG-CD44cyt (by GTPγS•GST-RhoA-activated ROK). B: Scachard plot analysis of the ^{125}I-Ankyrin binding to minimally phosphorylated FLAG-CD44cyt (by GTPγS•GST-RhoA-treated ROK in the presence of staurosporine). C: The total amount of ^{125}I-Ankyrin binding to highly phosphorylated FLAG-CD44cyt (by GTPγS•GST-RhoA-activated ROK) (a) or minimally phosphorylated FLAG-CD44cyt (by GTPγS•GST-RhoA-treated ROK in the presence of staurosporine) (b).

Previous studies indicate that $CD44v_{3,8-10}$-mediated invasive phenotype of breast tumor cells (e.g. Met-1 cells) characterized by an "invadopodia" structure (or membranous projections) and tumor cell migration is closely associated with ankyrin-linked cytoskeleton function (Bourguignon et al., 1998). Ankyrin is a family of membrane-associated cytoskeletal proteins expressed in a variety of biological systems including epithelial cells and tissues (Peters and Lux, 1993). Presently, at least three ankyrin genes have been identified: ankyrin 1 (Ank 1 or ankyrin R), ankyrin 2 (Ank 2 or ankyrin B) and ankyrin 3 (Ank 3 or ankyrin G) (Lux et al., 1990; Otto, et al., 1991; Kordeli, et al., 1995; Peters, et al., 1995). These molecules belong to a family of related genes that probably arose by duplication and divergence of a common ancestral gene. Using specific anti-Ank1 and anti-Ank3 antibody, we have identified the presence of at least two ankyrin species (e.g. Ank1 and Ank3) in breast tumor cells such as Met-1 (Fig. 7). In particular, we have found that microinjection of CAT domain (Fig. 7A-D) promotes CD44 (e.g. $CD44v_{3,8-10}$) (Fig. 7A and 7C) co-localization with ankyrin [e.g. Ank1 (Fig. 7B) and AnK3 (Fig. 7D)] in the plasma membranes and membrane projections (Fig. 7A-7D). In contrast, treatment of Met-1 cells (microinjected with CAT domain) with rabbit anti-CD44v3 antibody (specific for v3 sequence) (Fig. 7a and 7b) or cytochalasin D, the microfilament inhibitor (Fig. 7c and 7d) blocks CD44 (Fig. 7a and 7c) and ankyrin (Fig. 7b and 7d)-associated membrane projections. Cells that are not injected (Fig. 7e and 7f) and COS cells that do not express CD44 but were injected with CAT of ROK (Fig. 7g and 7h) fail to display active membrane motility (e.g. membrane projections). Moreover, our data show that CD44-ankyrin-associated membrane projections is significantly inhibited in Met-1 cells microinjected with RB domain (Fig. 7E and 7F). Together, these findings suggest that ROK (in particular CAT domain but not RB domain)-activation and ankyrin-CD44v3 signaling are closely coupled in Met-1 cell membrane motility.

Furthermore, using in vitro migration assays, we have found that $CD44v_{3,8-10}$-containing Met-1 cells undergo active cell migration (Table 1). Treatments of Met-1 cells with various agents such as anti-$CD44v_3$ antibody and cytochalasin D, cause a significant inhibition of tumor cell migration (Table 1). Importantly, transfection of Met-1 cells with the dominant-negative form of ROK cDNA (e.g. GFP-RB cDNA) but not vector alone (pEGFPC1 vector) also effectively blocks $CD44v_{3,8-10}$-cytoskeleton-dependent Met-1tumor cell motility (Table 1). Together, these findings suggest that $CD44v_{3,8-10}$, cytoskeleton and ROK must be functionally linked during metastatic breast tumor progression.

KEY RESEARCH ACCOMPLISHMENTS

- We have determined that $CD44v_{3,8-10}$ and RhoA GTPases are physically associated in metastatic breast tumor cells (Met-1 cell line).
- $CD44v_{3,8-10}$-bound RhoA displays GTPase activity which can be inhibited by botulinum toxin C3-mediated ADP-ribosylation.
- We have also identified a 160kDa Rho-Kinase (ROK) as one of the downstream targets for $CD44v_{3,8-10}$-bound RhoA GTPase in metastatic breast tumor cells.
- Our results show that RhoA (complexed with $CD44v_{3,8-10}$) stimulates ROK-mediated phosphorylation of certain cellular proteins including the cytoplasmic domain of $CD44v_{3,8-10}$.
- It is noted that phosphorylation of $CD44v_{3,8-10}$ by ROK enhances its interaction with the cytoskeletal protein, ankyrin.
- We have constructed two ROK cDNA constructs which encode for proteins consisting of 537 amino acids [designated as the constitutively active form of ROK containing the catalytic domain (CAT, also the kinase domain)], and 173 amino acids [designated as the dominant-negative form of ROK containing the Rho-binding domain (RB)].
- Our results indicate that microinjection of the ROK's CAT domain into metastatic breast tumor cells promotes CD44-ankyrin associated membrane ruffling and projections. This membrane motility can be blocked by CD44 antibodies and cytochalasin D (a microfilament inhibitor).
- We have found that overexpression of a dominant-negative form of ROK by transfection of metastatic breast tumor cells with ROK's Rho-binding (RB) domain cDNA effectively inhibits CD44-ankyrin-mediated metastatic behavior (e.g. membrane motility and tumor cell migration).
- These findings support the hypothesis that ROK plays a pivotal role in $CD44v_{3,8-10}$-ankyrin interaction and RhoA-mediated oncogenic signaling required for membrane-cytoskeleton function and metastatic tumor cell migration.

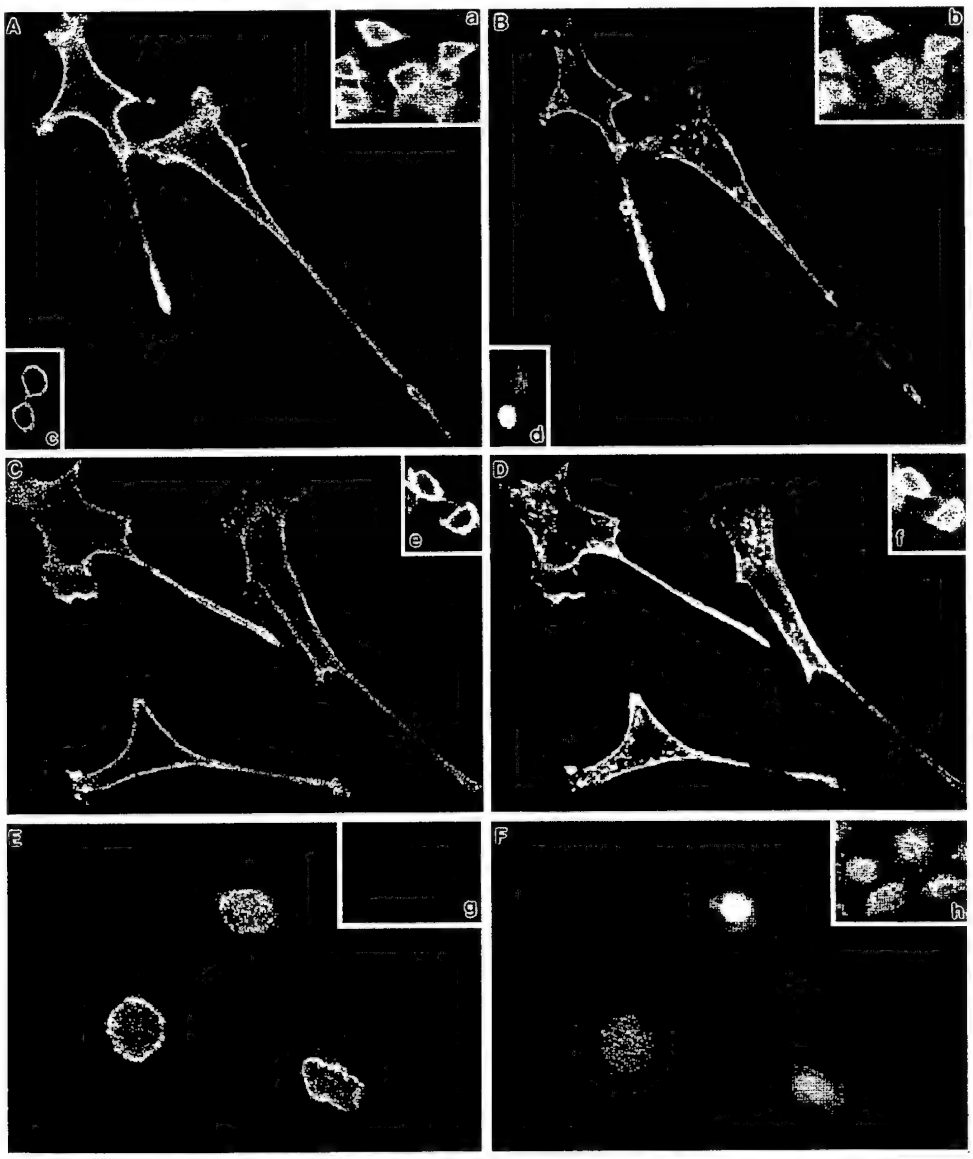

Fig. 7: ***Double immunofluorescence staining of CD44 and ankyrin in Met-1 cells microinjected with CAT or RB domain.***

Met-1 cells [microinjected with CAT or RB domain] grown in the presence and absence of certain agents [e.g. rabbit anti-CD44v3 antibody (50µg/ml) or cytochalasin D (20µg/ml)] were fixed by 2% paraformaldehyde. Subsequently, cells were surface labeled with FITC-labeled rat anti-CD44 antibody. These cells were then rendered permeable by ethanol treatment and stained with rhodamine (Rh)-labeled mouse anti-Ank1 IgG or Rh-labled rabbit anti-Ank3 IgG. To detect non-specific antibody binding, FITC-CD44-labeled cells were incubated with Rh-conjugated normal mouse IgG or Rh-conjugated rabbit preimmune serum. No labeling was observed in such control samples.

A-D: Staining of surface CD44 with FITC-conjugated rat anti-CD44 antibody (A and C); and intracellular ankyrin (Ank1) with Rh-conjugated mouse anti-Ank1 antibody (B) and Ank3 antibody (D) in Met-1 cells microinjected with CAT domain.

E & F: Staining of surface CD44 with FITC-conjugated rat anti-CD44 antibody (E); and intracellular ankyrin with Rh-conjugated mouse anti-Ank1 antibody (F) in Met-1 cells microinjected with RB domain.

a & b: Staining of surface CD44 with FITC-conjugated rat anti-CD44 antibody (a); and intracellular ankyrin with Rh-conjugated mouse anti-Ank1 antibody (d) in Met-1 cells microinjected with CAT domain and incubated with rabbit anti-CD44v3 antibody.

c & d: Staining of surface CD44 with FITC-conjugated rat anti-CD44 antibody (e); and intracellular ankyrin with Rh-conjugated mouse anti-Ank1 antibody (f) in Met-1 cells microinjected with CAT domain and incubated with cytochalasin D.

e & f: Staining of surface CD44 with FITC-conjugated rat anti-CD44 antibody (a); and intracellular Ank1 with Rh-conjugated mouse anti-Ank1 antibody (b) in Met-1 cells without any injection treatment.

g & h:Staining of surface CD44 with FITC-conjugated rat anti-CD44 antibody (g); and intracellular ankyrin with Rh-conjugated mouse anti-Ank1 antibody (h) in COS-7 cells (a CD44-negative cell line) microinjected with CAT domain.

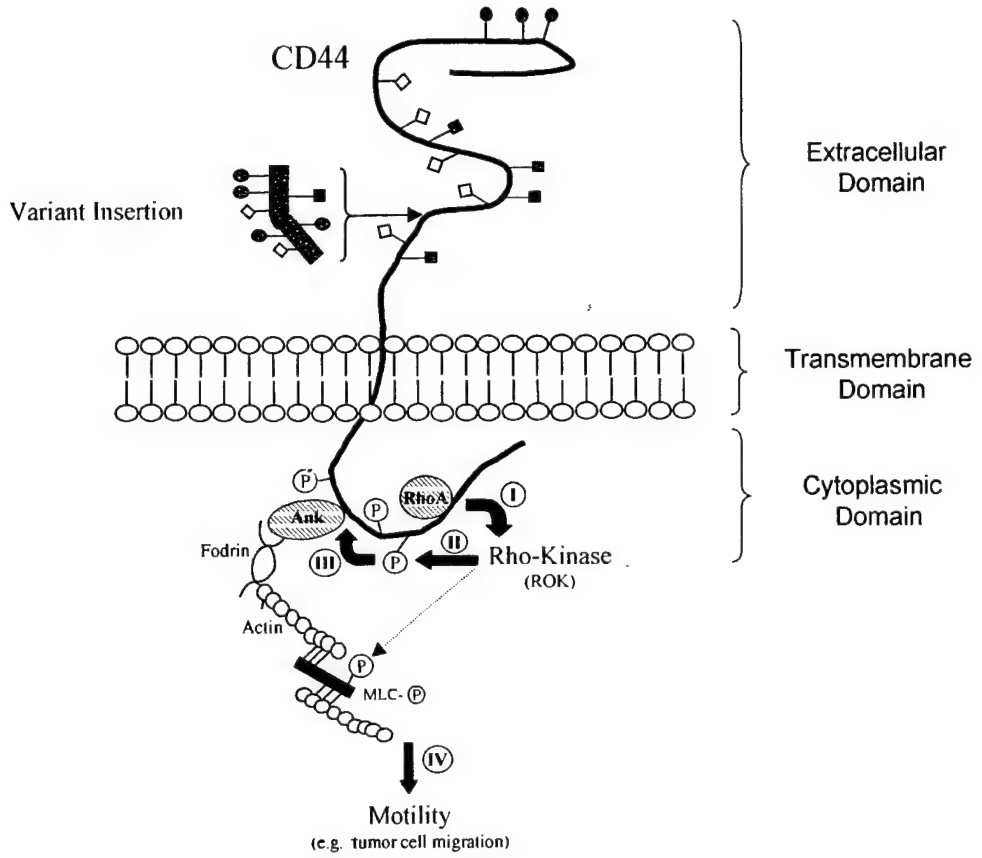

Fig. 8: ***A proposed model for the regulation of membrane-cytoskeleton interaction and cell motility in metastatic breast tumor cells by $CD44v_{3,8\text{-}10}$-RhoA signaling and ROK activation.***
Step I: $CD44v_{3,8\text{-}10}$ (containing oncogenic signaling-related v3 exon-encoded structure) is tightly coupled with RhoA in a complex which can up-regulate Rho-Kinase (ROK) activity.
Step II: Activated ROK phosphorylates cellular proteins including the cytoplasmic domain of $CD44v_{3,8\text{-}10}$ and possibly myosin light chain (MLC-P-shown by a thin dotted line).
Step III: Phosphorylation of $CD44v_{3,8\text{-}10}$'s cytoplasmic domain by ROK promotes the binding of $CD44v_{3,8\text{-}10}$ to the cytoskeletal protein, ankyrin (Ank), which in turn interacts with fodrin and actin-myosin-based microfilamenteous contractile elements.
Step IV: This membrane-cytoskeleton interaction is required for cell motility including tumor cell migration during breast cancer progression.

Table 1: Measurement of Tumor Cell Migration.

Cells	Cell Migration[a] (% of Control)[b]		
	No treatment	**Anti-CD44v3-treated**	**Cytochalasin D treated**
Untransfected cells (control)	100	27	24
Vector-transfected cells	98	25	22
GFP-RB cDNA-transfected cells	21	≤5	≤5

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a: Met-1 cells [≈ 1 x 10^4 cells/well in phosphate buffered saline (PBS), pH 7.2] [in the presence or absence of rabbit anti-CD44v3 antibody (50 μg/ml) or cytochalasin D (20 μg/ml)] were placed in the upper chamber of the transwell unit. In some cases, Met-1 cells were transfected with either GFP-tagged RB cDNA or GFP-vector alone. After 18h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (58). Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically by Student's t test and statistical significance was set at $p<0.01$. In these experiments ≈30 to 40% of input cells (≈ 1 x 10^4 cells/well) undergo in vitro migration in the control samples.
b: The values expressed in this table represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

REPORTABLE OUTCOMES

Paper Publications:

1. Kalish, E., N. Iida, F.L. Moffat and Lilly Y. W. Bourguignon. A New CD44v3-Containing Isoform Is Involved in Tumor Cell Migration and Human Breast Cancer Progression. Front Biosci. 4:1-8 (1999).
2. Ameen, N.A., B. Martensson, Lilly Y. W. Bourguignon, C. Marino, J. Isenberg and G.E. McLaughlin. CFTR Channel Insertion to The Apical Surface In Rat Duodenal Villus Epithelial Cell Is Upregulated By VIP In Vivo. J. Cell Sci. 112:887-894 (1999).
3. Bourguignon, Lilly Y.W., H.Zhu, L. Shao, D. Zhu and Y.W.Chen, Rho-Kinase (ROK) Promotes $CD44v_{3,8\text{-}10}$-Ankyrin Interaction And Tumor Cell Migration In Metastatic Breast Cancer Cells. Cell Motility & The Cytoskeleton, 43:269-287 (1999).
4. Bourguignon, Lilly Y.W., H.Zhu, L. Shao and Y.W.Chen, CD44 Interaction with Tiam1 Promotes Rac1 Signaling and Hyaluronic Acid (HA)-Mediated Breast Tumor Cell Migration. J. Biol. Chem. 275:1829-1838 (2000).
5. Zhu, D. and Lilly Y.W. Bourguignon. Interaction Between CD44 and The Repeat Domain of Ankyrin Promotes Hyaluronic Acid (HA)-Mediated Ovarian Tumor Cell Migration. J. Cell Physiol. 183:182-195 (2000).
6. Bourguignon, Lilly Y.W., H.Zhu, L. Shao and Y.W.Chen, Ankyrin-Tiam1 Interaction Promotes Rac1 Signaling and Metastatic Breast Tumor Cell Invasion and Migration. J. Cell Biol. 150:177-191 (2000).
7. Diaz, F. and Lilly Y.W. Bourguignon. Selective Down-Regulation of IP3 Receptor Subtypes By Caspases and Calpain During TNFα-Induced Apoptosis of Human T-Lymphoblasts. Cell Calcium 27:315-328 (2000).

Abstracts:

1. Bourguignon, Lilly Y.W., H.Zhu, L. Shao, and Y.W.Chen, Identification Of An Ankyrin-Binding Domain In TIAM1 And Its Role In Regulating $CD44v_{3,8\text{-}10}$-Associated Metastatic Breast Tumor Cell Invasion And Migration. Proceeding of the American Association for Cancer Res. 40:196 (1999).
2. Bourguignon, Lilly Y.W., H.Zhu, L. Shao, D. Zhu and Y.W.Chen, Rho-Kinase (ROK) Promotes $CD44v_{3,8\text{-}10}$-Ankyrin Interaction And Tumor Cell Migration In Metastatic Breast Cancer Cells. Proceeding of the American Association for Cancer Res. 40:105 (1999).
3. Zhu, D. and Lilly Y.W. Bourguignon. Interaction Between CD44 and The Repeat Domain of Ankyrin Promotes Hyaluronic Acid (HA)-Mediated Ovarian Tumor Cell Migration. Proceeding of the American Association for Cancer Res. (2000).
4. Bourguignon , Lilly Y.W., H.Zhu, L. Shao and Y.W.Chen, CD44 Interaction with Tiam1 Promotes Rac1 Signaling and Hyaluronic Acid (HA)-Mediated Breast Tumor Cell Migration. Proceeding of the American Association for Cancer Res. (2000).
5. Bourguignon, Lilly Y.W., H.Zhu, L. Shao and Y.W.Chen, Ankyrin-Tiam1 Interaction Promotes Rac1 Signaling and Metastatic Breast Tumor Cell Invasion and Migration. Mol. Biol. Cell (2000).
6. Singleton, P.A. and Bourguignon, Lilly Y.W.CD44v10 Interaction with Rho-Kinase (ROK) Promotes Cytoskeleton Function and HA-Mediated Endothelial Cell migration. Mol. Biol. Cell (2000).

•Funding applied for based on work supported by this award:

Funded Active Grants:

1. NCI Grant (2000-2005)"CD44/Variant-Cytoskeleton In Breast Cancer Progression".
2. NCI Grant (1999-2003) "CD44-$p185^{HER2}$ Interaction In Ovarian Cancer Progression".
3. US Army Breast Cancer Grant (DOD) (1999-2002) "A Novel Signaling Perturbation and Ribozyme Gene Therapy Proceudre to Block Rho-Kinase (ROK) Activation and Breast Tumor Metastasis".

CONCLUSIONS

CD44 [CD44 standard form (CD44s) and variant isoforms (CD44v)] belongs to a family of transmembrane glycoproteins known to bind extracellular matrix components [e.g. hyauronic acid (HA)] in its extracellular domain and interact with the cytoskeletal protein, ankyrin, at its cytoplasmic domain (Bourguignon, 1996; Lesley et al., 1993). Cells expressing a high level of CD44 isoforms often display enhanced hyaluronic acid binding which is directly related to tumor cell growth and migration (Zhang et al., 1995). HA binding to CD44s has been shown to stimulate the $p185^{HER2}$-associated tyrosine kinase which is linked to CD44s via a disulfide linkage (Bourguignon et al., 1997), and results in direct "cross-talk" between two different signaling pathways (e.g. proliferation vs motility/invasion)(Bourguignon et al., 1998). Most importantly, certain angiogenic factors and matrix degradating enzymes (MMPs) are also tightly associated with CD44v isoforms (Bourguignon et al., 1998), and are believed to play synergistic roles in the generation of oncogenic signals leading to tumor-specific behaviors (e.g. invasion and motility/migration) in a cytoskeleton-dependent manner (Bourguignon et al., 1998).

As the histologic grade of each tumor progresses, the percentage of lesions expressing CD44 variant isoforms increases. In particular, the CD44v3-containing isoforms (e.g. $CD44v_{3,8\text{-}10}$) are expressed preferentially on highly malignant breast carcinoma tissue samples (Bourguignon et al., 1995; Bourguignon, 1996; Iida and Bourguignon, 1997) and metastatic breast tumor cells (Fig. 1) (Bourguignon et al., 1998). In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (Bourguignon et al., 1995; Iida and Bourguignon, 1997, Sinn et al., 1995). One study indicates that expression of the CD44v3 isoform in breast tumors may be used as an accurate predictor of overall survival (Kaufmann et al., 1995). Previously, $CD44v_{3,8\text{-}10}$ has been shown to contain sulfated oligosaccharides (Bennet et al., 1995; Jackson et al., 1995). Our recent results indicate that $^{35}SO_4^{2-}$ is incorporated into the glycosaminoglycan (GAG) chains of $CD44v_{3,8\text{-}10}$ isolated from Met-1 cells (Bourguignon et al., 1998). The GAG chains of CD44v3-containing isoforms appear to be important in the linkage of heparin binding growth factors (Bennet et al., 1995; Bourguignon et al., 1998; Jackson et al., 1995). For example, $CD44v_{3,8\text{-}10}$ of Met-1 cells binds preferentially to vascular endothelial growth factor (VEGF), but not basic fibroblast growth factor (bFGF) (Bourguignon et al., 1998). VEGF is a specific mitogen for endothelial cells and a potent microvascular permeability factor (Dvorak et al., 1995; Folkman, 1985). It plays an integral role in angiogenesis and thus in potentiation of solid tumor growth (Dvorak et al., 1995; Folkman, 1985). Therefore, the attachment of VEGF to the heparin sulfate sites on $CD44v_{3,8\text{-}10}$ may be responsible for the onset of breast tumor-associated angiogenesis. It is also speculated that some of these CD44v3 isoforms on epithelial cells may act as surface modulators to facilitate unwanted growth factor receptor-growth factor interactions (Bennet et al., 1995; Bourguignon et al., 1998; Jackson et al., 1995) and subsequent tumor metastasis. Recently, we have found that the $CD44v_{3,8\text{-}10}$ isoform expressed on Met-1 cell surface is closely associated with the matrix metalloproteinase, MMP-9, and interacts with the cytoskeleton to promote tumor cell-specific phenotypes including "invadopodia" formation and tumor cell migration (Bourguignon et al., 1998). These findings suggest that $CD44v_{3,8\text{-}10}$ and its associated cytoskeleton play an important role in metastatic tumor cell behavior.

Cytoskeletal reorganization has been linked to the activation of Rho-like proteins including RhoA, Rac1 and Cdc42 (Hall, 1998; Narumiya, 1996). Specifically, RhoA is required for actin filament bundling, stress fiber formation and acto-myosin-based contractility (Hall, 1998; Narumiya, 1996). In this study we have demonstrated that RhoA (but not Rac or Cdc42) is closely associated with $CD44v_{3,8\text{-}10}$ as a complex (Fig. 1). RhoA complexed with $CD44v_{3,8\text{-}10}$ binds GTP and displays GTPase activity which can be inhibited by C3 toxin-mediated ADP-ribosylation (Fig. 2). In order to establish the functional involvement of this $CD44v_{3,8\text{-}10}$-RhoA complex in regulating the metastatic phenotype, we have searched for the downstream effector(s) for this transmembrane complex. Rho-Kinase (ROK) (also called Rho-binding Kinase) is known to bind Rho GTPase and participate in cytoskeleton functions and membrane motility (Amano et al., 1997; Leung et al., 1996; Matsui et al., 1996). ROK also phosphorylates myosin light chain phosphatase and myosin light chain (Amano et al., 1996; Kimura et al., 1996), thereby activating myosin adenosine triphosphatase (ATPase) and generating actomyosin-mediated

membrane motility (Amano et al., 1996; Kimura et al., 1996). ROK is also responsible for the phosphorylation of CD44-associated cytoskeletal proteins [e.g. Ezrin/Radixin/Moesin (ERM)] during actin filament and plasma membrane interaction (Matsui, et al., 1998). When ROK is overexpressed or constitutively activated, changes in actin cytoskeleton organization occur which are similar to those observed during Rho-activated conditions (Kimura et al., 1996; Leung et al., 1996). This evidence prompted us to investigate ROK as a possible downstream effector for RhoA (complexed with $CD44v_{3,8\text{-}10}$). In Met-1 cells, we have identified a 160 kDa protein as a ROK-like protein containing RhoA-binding properties (Fig. 3B). This 160kDa ROK activity can be activated by binding to activated RhoA but not unactivated RhoA (Figs. 4 and 5). Both 160 kDa ROK and the catalytic domain (GST-CAT) (but not the Rho-binding domain, GFP-RB) of ROK appear to be essential for activating ROK-mediated phosphorylation of cellular proteins including myosin light chain (Fig. 4A), $CD44v_{3,8\text{-}10}$ (Fig. 4B) and the cytoplasmic domain of CD44 (Figs. 4C and 5). These results clearly indicate that ROK acts as one of the downstream effectors of RhoA (complexed with $CD44v_{3,8\text{-}10}$).

Ankyrin (e.g. Ank1, Ank2 or Ank3) is known to bind to a number of plasma membrane-associated proteins including band 3, two other members of the anion exchange gene family (Bennet, 1992), Na^+/K^+-ATPase (Zhang et al., 1998), the amiloride-sensitive Na^+ channel (Smith et al., 1991), the voltage-dependent Na^+ channel (Kordeli et al., 1995), Ca^{2+} channels (Bourguignon et al., 1993; Bourguignon and Jin, 1995 Bourguignon et al., 1995) and the adhesion molecule, CD44 (Bourguignon et al., 1995; Bourguignon, 1996, Bourguignon et al., 1997). It has been suggested that the binding of ankyrin to certain membrane-associated molecules may be needed for signal transduction, cell adhesion, cell migration and tumor metastasis (Bourguignon et al., 1993; Bourguignon et al., 1995). The cytoplasmic domain of CD44s (approximately 70 a. a. long) is highly conserved ($\approx$ 90%) in most of the CD44 isoforms, and is clearly involved in specific ankyrin binding (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). The ankyrin-binding domain of CD44 has also been mapped using deletion mutation analyses and mammalian expression systems (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). The results of these experiments indicate that at least two subregions within the CD44 cytoplasmic domain constitute the ankyrin binding-region I (i.e. the high affinity ankyrin-binding region) and region II (i.e. the regulatory region). In particular, the region I ankyrin-binding domain (e.g. "NGGNGTVEDRKPSEL" between aa 306 and aa 320 in the mouse CD44 and "NSGNGAVEDRKPSGL " aa 304 and aa 318 in human CD44) is required for cell adhesion, Src kinase recruitment and tumor cell transformation (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). Recent data indicate that the amino acid sequence-"NGGNGTVEDRKPSEL" [located between aa 480 and aa 494 of $CD44v_{3,8\text{-}10}$] in Met-1 cells binds specifically to the cytoskeletal protein, ankyrin (but not fodrin or spectrin), and belongs to the ankyrin-binding domain of $CD44v_{3,8\text{-}10}$ (Bourguignon et al., 1998).

Previously, it has been shown that the binding interaction between CD44 and ankyrin is up-regulated by several factors including protein kinase C-mediated phosphorylation (Bourguignon et al., 1992, Kalomiris and Bourguignon), palmityolation (Bourguignon et al., 1991) and GTP-binding (Hirao et al., 1996; Lokeshwar and Bourguignon, 1992). In this study we have provided new evidence that phosphorylation of CD44 by RhoA-activated ROK also stimulates its binding to ankyrin (Fig. 6). Further analyses using double immunofluorescence staining show that Met-1 cells express both Ank1 (Fig. 7 B) and Ank3 (Fig. 7 D). In particular, the catalytic domain (CAT) but not the Rho-binding domain (RB) of ROK is responsible for promoting CD44 phosphorylation (Figs. 4 and 5) which up-regulates CD44-ankyrin (e.g. Ank1 and/or Ank3)-mediated membrane motility (Fig.7 A-D). Moreover, Met-1 tumor cell migration is inhibited by cytochalasin D (Table 1), suggesting that some actin polymerization or microfilamenteous cytoskeleton might be required. Our findings are consistent with a previous study indicating CD44 and its associated cytoskeletal proteins [e.g. ERM (ezrin, radixin and moesin)-F-actin complex] appear to be an essential prerequisite for the Rho GTPase-induced cytoskeletal changes (Hirao et al., 1996;Tsukita et al., 1994). This membrane-cytoskeleton interaction could also lead to abnormal tumor cell motility (e.g. membrane projections and cell migration) (Fig. 7 A-D; and Table 1) in Met-1 cells. In addition, we have found that treatment of Met-1 cells with anti-CD44v3 antibody/cytochalasin D (Fig. 7 a-d) or overexpression of the Rho-binding (RB) domain of ROK (by transfecting Met-1 cells with RbcDNA) (Fig. 7E

and F) induces reversal of tumor cell-specific phenotypes such as membrane motility and tumor cell migration (Table 1). Together, these findings strongly suggest that oncogenic signaling (derived from the v3-coded structure of $CD44v_{3,8-10}$) and ROK activation are closely coupled during the onset of cytoskeleton function and tumor-specific behavior in metastatic breast tumor cells. As summarized in Fig. 8, we propose that $CD44v_{3,8-10}$ (containing oncogenic signaling-related v3 exon-encoded structure) is tightly coupled with RhoA in a complex which can up-regulate Rho-Kinase (ROK) activity (Step I). Activated ROK then phosphorylates certain cellular proteins including the cytoplasmic domain of $CD44v_{3,8-10}$ and possibly myosin light chain (Step II). Most importantly, phosphorylation of $CD44v_{3,8-10}$'s cytoplasmic domain by ROK promotes the binding of $CD44v_{3,8-10}$ to the cytoskeletal protein, ankyrin (e.g. Ank1 and/or Ank3), (Step III) which in turn interacts with fodrin and actin-myosin based microfilaments. We proposed that this membrane-cytoskeleton interaction is required for stimulating membrane motility and tumor cell migration (Step IV) during breast cancer progression.

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